Preparation and Characterization of Hydroxyapatite-Chitosan-Alginate micro-thin nanocomposites

Thesis submitted

in partial fulfillment as a requirement for the degree of

Bachelor of Technology

Submitted by:

Tanaya Roy

Roll No: 110CR0602

Under the Supervision

Of

Prof. Debasish Sarkar

Department of Ceramic Engineering
National Institute of Technology

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ROURKELA

CERTIFICATE

This is to certify that the project entitled "**Preparation and Characterization of Hydroxyapatite-Chitosan-Alginate Microthin Nanocomposites**" submitted by **Tanaya Roy (Roll No. 110CR0602)** is a genuine work performed by her under my guidance required for the **Bachelor of Technology** degree in **Ceramic Engineering** at National Institute of Technology, Rourkela.

To the best of my knowledge, this thesis is very authentic and none of its matter has been submitted anywhere else for the award of degree or diploma.

Date: 05/05/2014

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Also thanks to all the PhD scholars and M.Tech students who were always ready with their helpful hands and made this project a successful one.

And lastly thanks to all my friends who backed me up with their moral support and always motivated me throughout the project.

Tanaya Roy

110CR0602

Abstract

Recently, biomaterials have become an important area of research. In this research, tissue engineering is of utmost importance as it addresses some important biomedical issues. In the present study, we demonstrate the use of nano-hydroxyapatite, sodium alginate and chitosan as materials for skin tissue engineering and wound healing applications. In this method, films of thickness about 100 microns are fabricated through the mechanism of cross linking through $CaCl_2$ solution. Calcium ions replace sodium ions in the alginate and form a gel film upon air drying. The films are then tested for various properties like bioactivity, biodegradability, tensile strength, porosity to understand the suitability of the material for replacement of skin. It has been found that the micro-thin composite shows good bioactivity and biodegradability. Tensile strength of the composite having 0.25% hydroxyapatite is 22.52N/mm^2. This proves to be an acceptable matrix as our natural skin has tensile strength in the range between 15-20N/mm^2. XRD shows the presence of HAP with respect to other added polymers. FESEM result shows a fairly good distribution of the HAP into the composite matrix along with good porosity that is required for tissue ingrowth.

Contents

Chapter-1

Introduction

Biomaterials are materials which have indispensible use in the field of medicine. Some of these biomaterials incorporate the use of hydroxyapatite. This hydroxyapatite, in the nano size, can be used for tissue engineering applications as it is a bioactive material. A bioactive material is one which when used as an artificial tissue makes a chemical bond with the natural tissue thus strengthening the implants. The properties which the biomaterial should have for skin tissue engineering are: it should be injectible and self-setting, either in the gel or scaffold form, it should be biodegradable so that the biomaterial degrades in vivo and new skin is formed, it should be biocompatible and not generate immunity responses, it should have tensile strength near to the tensile strength of the skin, it should be flowable and have enough viscosity not to overflow but to fill the wound and finally it should be bioactive. The polymer used is a combination of alginate and chitosan which could serve as a matrix for the skin cells to grow and degrade to give new skin. Alginate is chosen due to its good gelation properties with Ca^{2+} ions.

Hydroxyapatite

Hydroxyapapite is naturally occurring with the formula $(Ca_{10}(PO_4)_6(OH)_2$ which is the main inorganic element of the human bone. It is the hydroxyl end member of the apatite group which can be replaced by fluoride, chloride or carbonate. The pure hydroxyapatite powder is white. Hydroxyapatite is similar to the mineral component of bones and hard tissues in mammals. In implants, it integrates in bone structures and supports bone ingrowth i.e, shows its bioactive nature. Hydroxyapatite coatings are often applied to metallic implants to alter the surface properties [1]. The body recognizes hydroxyapatite-type material and accepts it without reactions. Without it, the body sees a foreign body and works in such a way as to isolate it from surrounding tissues thus causing various reactions. These may arise when large sections of bone have had to be removed (e.g. bone cancers) or when bone augmentations are required (e.g

maxillofacial reconstructions or dental applications). The bone filler provides a scaffold and encourages the rapid filling of the pores of the scaffold by naturally forming bone and disintegrating which provides an alternative to bone grafts. Nanosized HAp, with a grain size less than 100 nm , has high surface activity and an ultrafine structure, similar to the mineral found in hard tissues. It is well known that bioceramics that mimic the bone mineral in composition and structure can more readily promote osteointegration and subsequent bone tissue formation [2].

Alginate

Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed which has been used for many biomedical applications, due to its biocompatibility, low toxicity, relatively low cost, and mild gelation by addition of divalent cations such as Ca^{2+} [3]. Figure 1 represents the structure of sodium alginate:

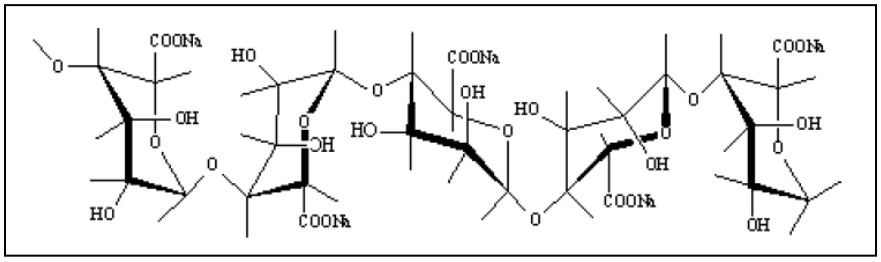


Figure 1: Structure of Sodium Alginate

Commercially available alginate is extracted from brown algae by treatment with aqueous alkali solutions, typically with NaOH. The extract is filtered, and either sodium or calcium chloride is added to the filtrate in order to precipitate alginate. After further purification and conversion, water-soluble sodium alginate powder is produced [3]. Alginate is typically used in the form of a hydrogel in biomedicine, including wound healing, drug delivery and tissue engineering

applications. These are biocompatible, being structurally similar to the macromolecular-based components in the body, and can often be delivered into the body via invasive administration. Cross-linking is usually done by chemical or physical cross-linking. The most common method to prepare hydrogels is to combine the solution with ionic cross-linking agents, such as divalent cations (i.e., Ca^{2+}). Calcium chloride ($CaCl_2$) is the most frequently used agent to cross-link alginate though it leads to rapid and poorly controlled gelation.

Alginate has been used in many biomedical applications including pharmaceutical fields like serving as thickening, gel forming or stabilizing agents for drug delivery applications. Alginate has also been researched in combination with chitosan, as the combination forms ionic complexes.

Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) [4]. It is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi. Figure 2 represents the structure of chitosan.

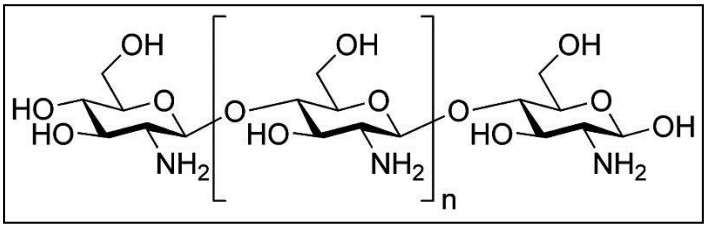


Figure 1: Structure of Chitosan

A common method for the synthesis of chitosan is the deacetylation of chitin using sodium hydroxide in excess as a reagent and water as a solvent. This reaction pathway, when allowed to go to completion (complete deacetylation) yields up to 98% product. Chitosan is water soluble and also a bio-adhesive which readily binds to negatively charged surfaces such as mucosal membranes. It also enhances the transport of polar drugs across epithelial surfaces, and is biocompatible and biodegradable.

The property of Chitosan allows it to rapidly clot blood and gain approval for use in bandages and other hemostatic agents that are used in tests to quickly stop bleeding and reduce blood loss. The chitosan salts can be mixed with other materials to make them more absorbent (such as mixing with alginate), or to vary the rate of solubility and bio-absorbability of the chitosan salt. Chitosan can be used to transport a drug to an acidic environment, where the chitosan packaging will then degrade, releasing the drug to the desired environment. One example has been the transport of insulin.

Chapter-2

Literature Survey

In tissue engineering, alginate has been researched extensively for bone tissue engineering due to its various properties. But alginate having low mechanical properties has been used with nano-hydroxyapatite, which is the mineral component of bones and is bioactive in nature. Alginate has also been used with chitosan to form complexes. In one study, a three-dimensional, porous alginate/HAP scaffold was prepared and characterized. The scaffold was prepared by a three-step procedure: initially gelation of the alginate-HAP solution was done to form a hydrogel with divalent cations (Ca^{2+}, Sr^{2+}, and Ba^{2+}), then freezing, and finally dried by lyophilization to produce a three-dimensional, porous sponge[7]. The highly porous, well-interconnected pore structure of the scaffold was developed through phase separation. The system contained two phases after phase separation: a polymer-poor and a polymer-rich phase. It was found out that pore architecture in alginate scaffolds can greatly affect the morphology of hepatocytes seeded within three-dimensional scaffolds [7].

In another study, porous hydroxyapatite (HAp)/chitosan-alginate composite scaffolds are prepared through in situ co-precipitation and freeze-drying for bone tissue engineering[8]. The composite scaffolds are highly porous and interconnected with a pore size of around 50-220 μm at low concentrations of HAp. As the HAp content increased, the porosity of the scaffolds decreased from 84.98 to 74.54% [8]. Chitosan and alginate, the polymer matrix of the composite scaffolds, were cross-linked, and the synthesized HAp was low crystallized. Porosity is based on the presence of open pores which are related to properties such as permeability and surface area of the porous structure. High porosity usually means a high surface area/volume ratio, and thus favors cell adhesion to the scaffold and promotes bone tissue regeneration. As the HAp content increased, the porosity of the composite scaffolds decreased, and the density increased [8]. An MTT assay indicates that the obtained scaffolds have no cytotoxic effects on MG-63 cells, and

that they showed good bio-compatibility [8]. Invivo compatibility was tested in which no inflammatory signs and adverse tissue reactions were seen. After 8 weeks of implantation, the site implanted with the composite scaffold had formed sufficient bone to span most of the bone defect, even though the control site had just observed low-density mineralization [8].

Furthermore, that HAp/chitosan-alginate composite scaffold was shown to be more effective for new bone generation than chitosan-alginate scaffold. In a different method, Porous scaffolds composed of alginate (AG) and chitosan (CS) were fabricated by combining the formation of polyelectrolyte complex (PEC) with freeze-drying [9]. The AG scaffold was used as a framework with uniformly distributed and interconnected pore structure [9]. The chitosan or chitosan/hydroxyapatite (CS/HA) composite solution was introduced into the pores of AG scaffold to form PEC scaffolds. FT-IR, XRD and XPS analysis confirmed that CS or CS/HA were coated on the AG scaffold surface. Microstructure, porosity, mechanical strength and thermal stability of PEC scaffolds were also investigated [9]. The AG-CS PEC scaffold and Ca^{2+} crosslinked AG scaffold showed low average pore diameter and lower porosity than those of uncrosslinked AG scaffold. [9]. Moreover, compared to Ca^{2+} crosslinked AG scaffold, AG-CS PEC scaffold exhibited higher mechanical strength and better thermal stability. The reasons for the mechanical strength improvement of crosslinked scaffold could be strong ionic interactions. For Ca^{2+} crosslinked AG scaffold, the mechanical strength was obviously enhanced because Ca^{2+} has strong ionic interaction with COO^- in alginate chain [9].

Objective of the work:

- To prepare films of microthin nature from nano hydroxyapatite, alginate and chitosan.
- To characterize the micro-thin nanocomposites by physicochemical techniques (XRD FESEM and tensile strength)
- To understand the bioactivity and biodegradibilty to use them for skin biomedical applications.

Chapter-3

Experimental Procedure

After various trials, a standard method was chosen for crosslinking of the Hydroxyapatite-chitosan-alginate microthin-composite films. The standardized process for preparation is given as follows:

1. **Hydroxyapatite content**- Hydroxyapatite content was increased in various samples from 1-10% weight in 20 ml solution but samples with HA content showed a high brittleness after 5% which was not suitable for skin engineering applications. So, HA content was fixed at 1-5% weight. Hydroxyapatite was prepared in nanorod form. It was prepared by mixing aqueous solutions of $(CH_3COO)_2Ca$ and KH_2PO_4 with identical molar ratio of calcium and phosphorous atoms in spherical particles. Two solutions were then slowly dropped together within a glass beaker through vigorous stirring. The precipitated HAp was aged for 1 h, washed and oven dried [10]. The basic differences within two processes of making spherical particles and nanorods were the starting precursors and reaction medium [10]. The sphere particles were developed in basic (pH-10) medium, and rods in weak acidic (pH -4) condition[10]
2. **Sodium alginate content-** Proper crosslinking of the alginate only occurred after addition of 2% weight. So alginate content was fixed at 2%, 3% and 4%.
3. **Chitosan content-** With increase in chitosan content from 0-3% weight, again samples showed an increase in brittleness after 1%. So chitosan was fixed at 0-1%.
4. **Water content-** Water content was fixed at 20 ml.
5. **Selection of the crosslinker-** Any insoluble Calcium solution could be used for the crosslinking. $CaCl_2$ was finally used as it had a rapid rate of crosslinking which is essential for insitu skin tissue engineering.

6. **$CaCl_2$ solution concentration-** It was fixed at an optimum content upto 5% weight of 100 ml water because a higher amount of $CaCl_2$ showed an erratic crosslinking.
7. **Crosslinking duration-** It was fixed at 30 minutes. A decrease in duration showed improper and incomplete crosslinking while an increase in duration showed higher shrinkage after drying.
8. **Drying condition-** 2 types of drying were tried out- air drying and freeze drying, both for 24 hours. Freeze drying was done at -52° C and 300 torr pressure. The freeze dried sample showed a great amount of shrinkage than the air dried sample. Also freeze drying is not a feasible concept for a composite which has to be self-setting.
9. **Shape of the HAP used** – There are 3 kinds of HAP i.e. spherical, rod and fibrous. Spherical Hydroxyapatite has a high surface area which can help in greater cell proliferation but the strength and crystallinity provided will be low. Fibrous hydroxyapatite has a low surface area but high crystallinity. So rod HA was used with a surface area of around 217 m^2/g and intermediate strength and crystallinity.

1) After the standardization of the parameters, the process was standardized. For obtaining the optimum composition of the raw materials, varying combitions of alginate, chitosan and nano-hydroxyapatite were used.

2) The raw materials were weighed properly with a weight balance. Hydroxyapatite was varied from 1-5%, chitosan from 0 -1% and alginate at 2%, 3% and 4% in 20 ml distilled water beaker.

3) The materials were mixed properly in the beaker on a magnetic stirrer at 1000 rpm without heat as the polymers would have burnt out. Alginate acted as a dispersant.

4) Mixing was done for about 45 minutes till a homogenous suspension was obtained.

5) The suspension was then poured into an alumina substrate and tapping was done for the solution to fill the substrate properly.

6) 2 weight% of 100 ml $CaCl_2$ solution was prepared and added to this suspension until it was totally covered by the solution.

7) This suspension was then kept to crosslink for about 30 minutes.

8) The $CaCl_2$ solution was then washed out and a gel-like film was formed which was continuously washed with distilled water.

9) The film was then kept to dry in air for 24 hours.

10) After complete drying, the samples were tested for various properties.

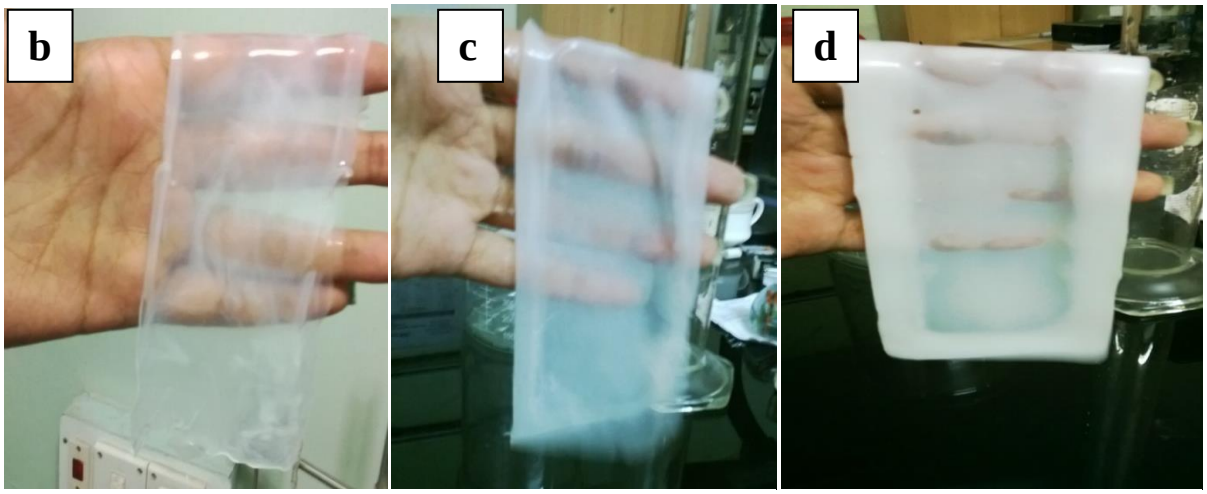
Table 1 shows the various compositions in weight percentage of HAP, chitosan and alginate.

Table1.

Sl. No.	HAP (weight%)	Chitosan (weight %)	Alginate (weight%)
1	0.5	0	2
2	1	0	2
3	1.5	0	2
4	2	0	2
5	2.5	0	2
6	0.5	0.5	2
7	1	0.5	2
8	1.5	0.5	2
9	2	0.5	2
10	2.5	0.5	2

11	0.5	1	2
12	1	1	2
13	1.5	1	2
14	2	1	2
15	2.5	1	2
16	0.5	0	3
17	1	0	3
18	1.5	0	3
19	2	0	3
20	2.5	0	3
21	0.5	0.5	3
22	1	0.5	3
23	1.5	0.5	3
24	2	0.5	3
25	2.5	0.5	3
26	0.5	1	3
27	1	1	3
28	1.5	1	3
29	2	1	3
30	2.5	1	3
31	0.5	0	4
32	0.5	0.5	4
33	0.5	1	4

Figure 3(a) shows the processing images during room temperature crosslinking of the microthin composite films with increase in HAP content. Figure 3(b), 3(c) and 3(d) shows the images of the same crosslinked films before drying (increase in HAP content).



Characterizations

1. XRD

XRD is an instrumental technique that is usually used to study the phase of the crystalline materials. The three dimensional structure of non-amorphous materials is defined by regular, repeating planes of atoms, part of the beam is transmitted, part is absorbed by the sample, part is refracted, scattered and part is diffracted. XRD methods for crystalline size determination are applicable for ranges from 2-100 nm. The diffraction peaks are broad for 2-3 mm crystallites while too small broadening for size above 100 nm. Scherrer equation is used for crystallite size determination. XRD of rod HAP, samples 5, 6, 10, 12, 19 were done in the present case.

2. FESEM

Field emission scanning electron microscopy operates via a field emission cathode in a scanning electron microscope providing narrower probing beams at low or high electron energy, which results in better spatial resolution and minimized sample charging and damage than the conventional SEM. FESEM of samples 5, 6, 10, 12 and 19 was done to see the particle distribution, pore size, pore size distribution, pore shape and interconnectivity.

3. Tensile strength

Tensile strength of the sample is the maximum elongation and tension required to break the composite. Usually, dog-bone shaped structures are used for this and the maximum load given by the universal testing machine is recorded. For tensile strength, fiber shaped samples were made to find out the diameter of the samples. They were made in an alumina boat and crosslinked at 4% weight $CaCl_2$ solution (higher than 2% used for films) and made to dry for 2 days. Then

testing was done with the help of the universal testing machine (UTM) which gave the amount of maximum load and maximum elongation at a speed of 0.5 mm/min. Figure 4 shows the Universal Testing Machine with the sample in the sample holder for tensile strength.

Figure 4: Universal Testing Machine

Tensile Strength= Max. Load / Area of cross section,

Where, Area of cross section = πr^2

4. Bioactivity

Bioactivity was measured in the SBF solution (Simulated body fluid). In the beginning, NaCl (99.5%), $NaHCO_3$ (99.5%), KCl (99.0%), $Na_2HPO_4.2H_2O$ (99.5%), $MgCl_2.6H_2O$(99.0%),

$Na_2SO_4.(CH_2OH)_3CNH_2$ (99.5%), $CaCl_2.H_2O$ (99.0%) and HCl (37 vol%) were used in the preparation of the SBF solution. Three pieces each of samples 5, 10, 12 and 19 were dipped into the solution and kept in an incubator at 37-40°C and pH of the SBF solution was maintained at 7.4. These samples were taken out in 5 days, 10 days, 15 days each and dried. FESEM of the samples were then done to find the attachment of the HA and interconnection of the bond.

5. Biodegrability

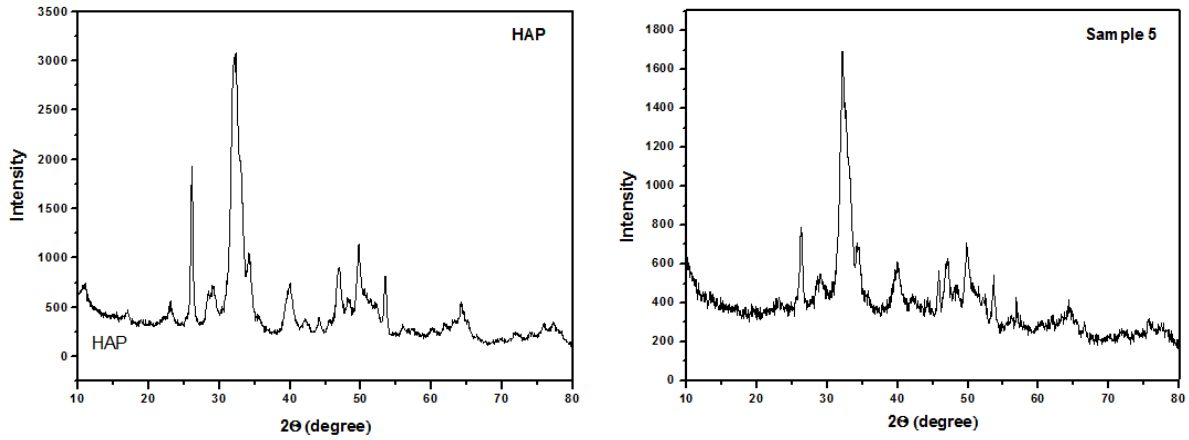
Biodegradibility of the samples are tested in 0.5 M tris-buffer solution. Dry weight of the samples 5, 10, 12 and 19 was first taken. Then it was dipped into this solution and kept in an incubator at 37-40°C and pH was maintained at 7.4. The samples are then taken out in 1 day, 3 days and 7 days, washed with distilled water and were dried and then dry weight taken. The percentage weight reduction was calculated.

Chapter-4

Results & Discussions

4.1. XRD Analysis

The X-ray diffraction patterns of various samples are plotted and analyzed. The graphs are given below in Figure 5. The graph with HAP showed sharp peaks at 2θ = 30° because of the crystallinity of the material. In Sample 5, small peaks are obtained due to the presence of an amorphous polymer alginate whereas, HAp peak was found constant at 2θ=30°. In Sample 6, a hump was formed due to the presence of polymers, i.e, alginate and chitosan as well as due to decrease in HAP content from 2.5% to 0.5%. Sample 10 has fairly high Hap content but again chitosan content increased the hump and a broad peak was obtained. Similarly, Sample 12 containing a low HAP content, showed a very broad peak. But Sample 19 had an equal distribution of HAP, chitosan and alginate so a narrow peak at 2θ=30° and smaller broad peaks was obtained.



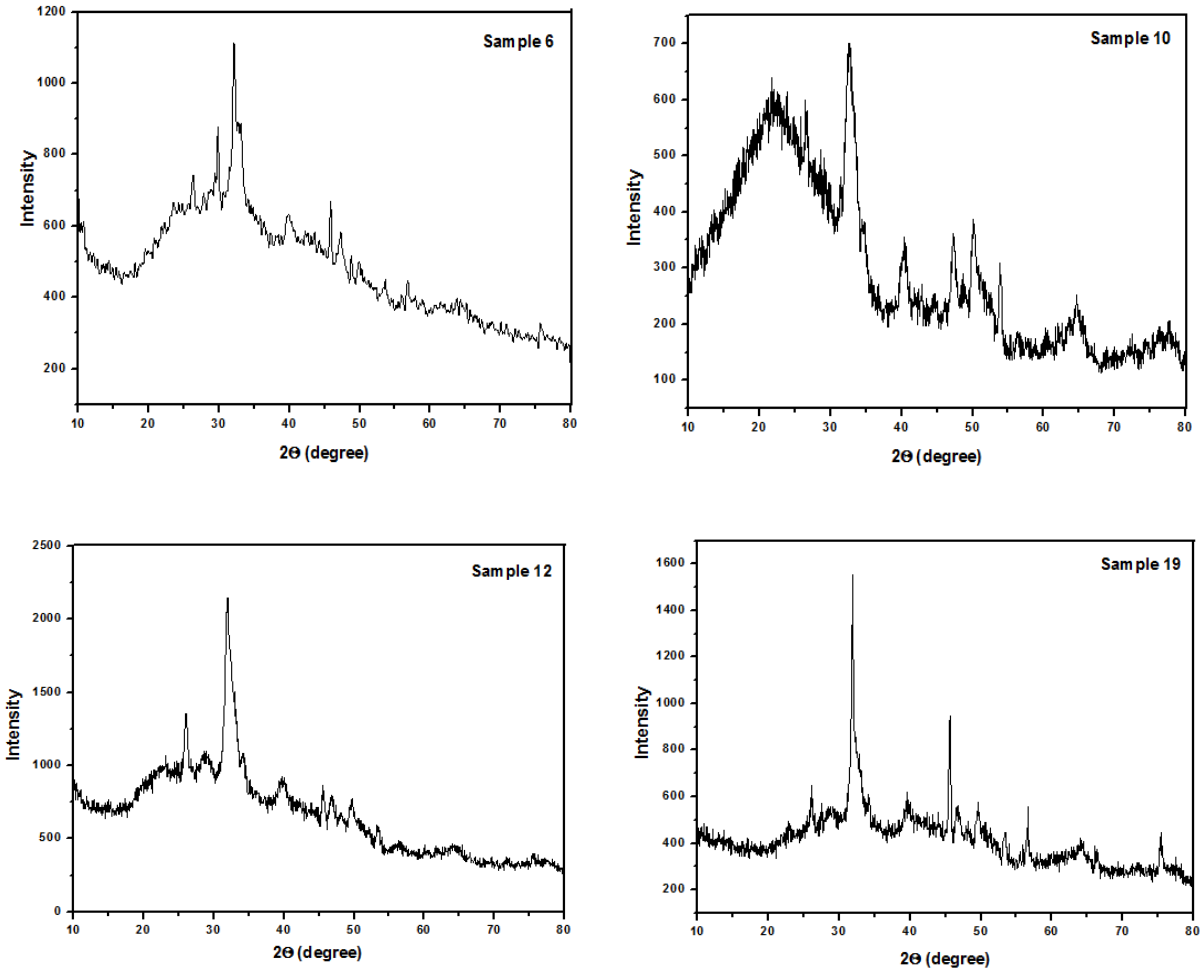
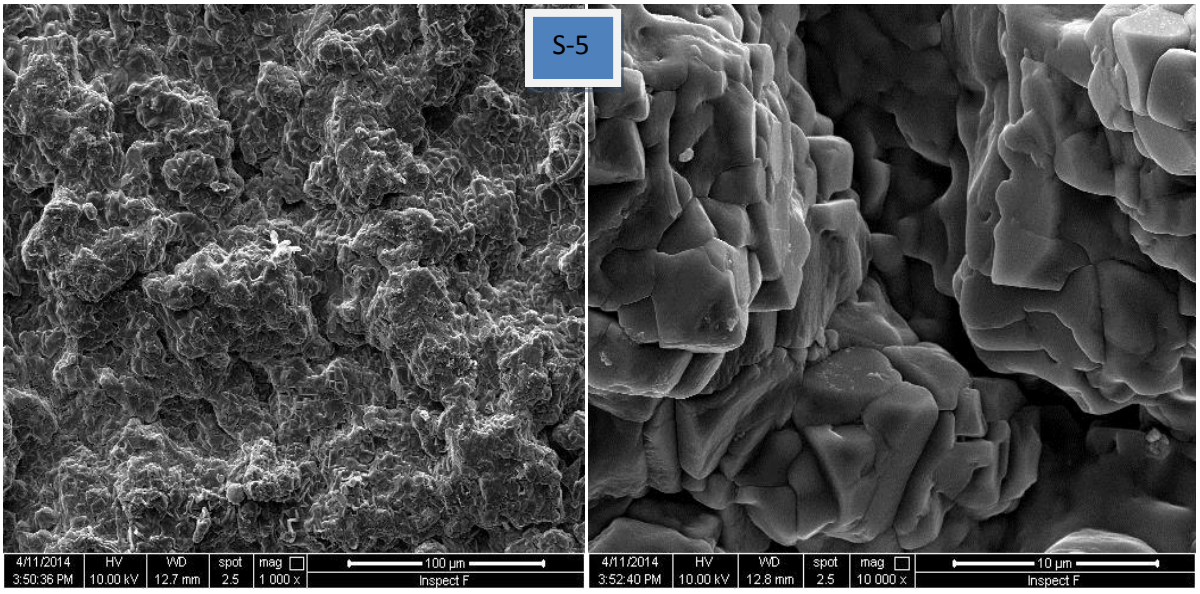
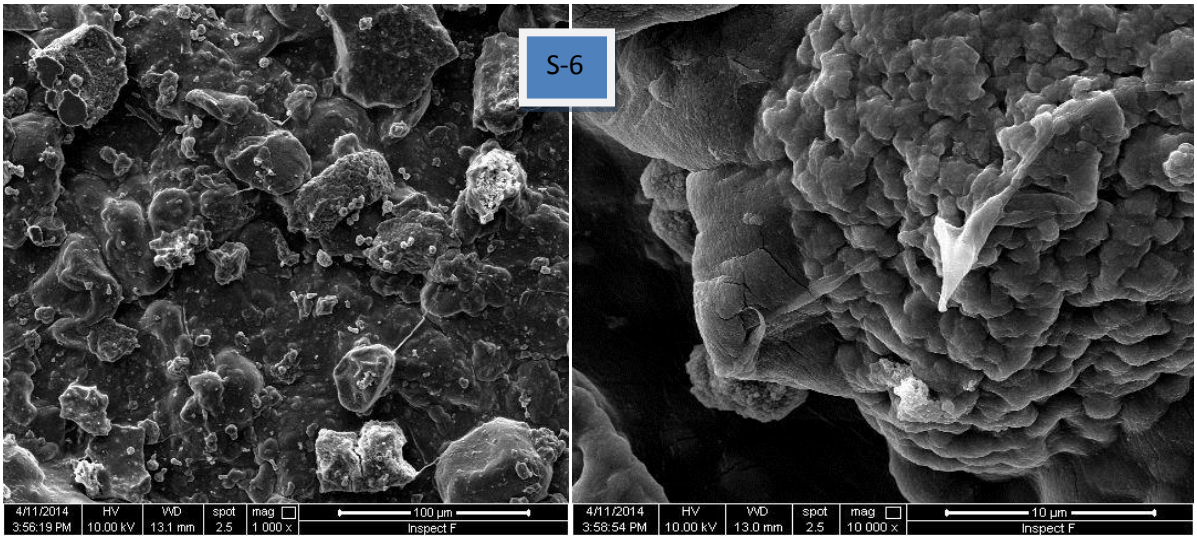

Sample 6
Intensity
2Θ (degree)
Sample 10
Intensity
2Θ (degree)
Sample 12
Intensity
2Θ (degree)
Sample 19
Intensity
2Θ (degree)

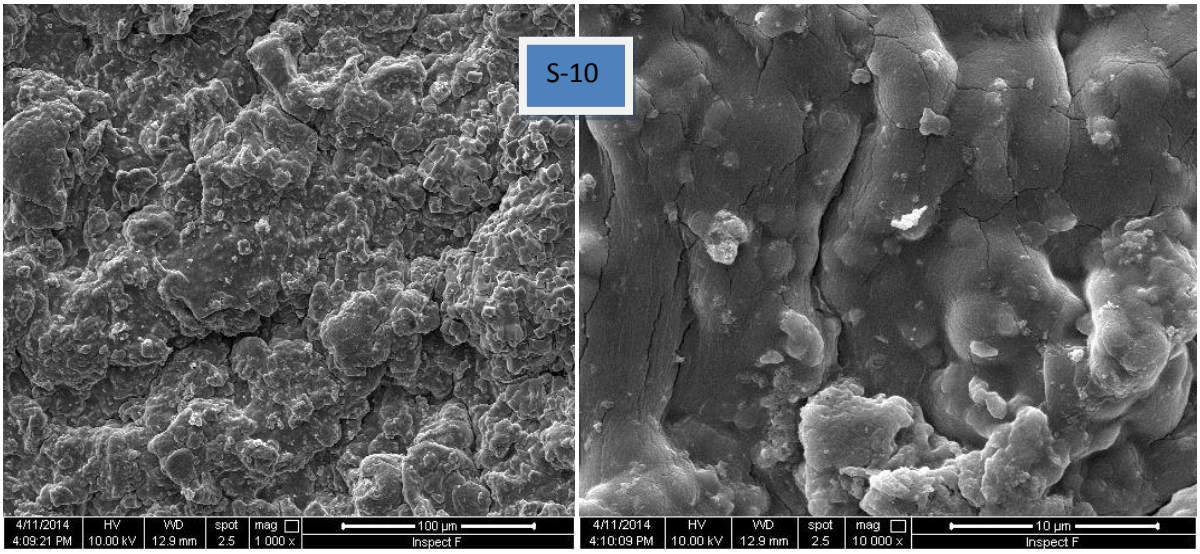
Figure 5

4.1. FESEM Analysis

The FESEM images of samples 5, 6, 10, 12, 19 are given below at 100 µm and 10 µm respectively. The images of all the samples showed a good homogeneity. The homogeneity in Sample 19 was rather found better with good pore structure. This can be found suitable for tissue ingrowth. The homogeneity and particle size distribution can be altered by process change but a good cell adhesion is required if used for wound healing applications.



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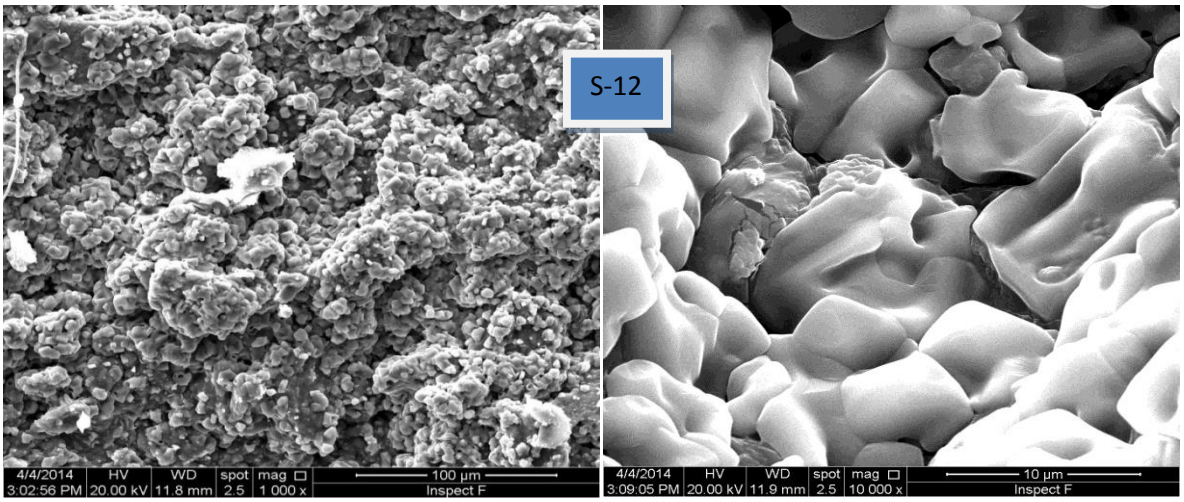
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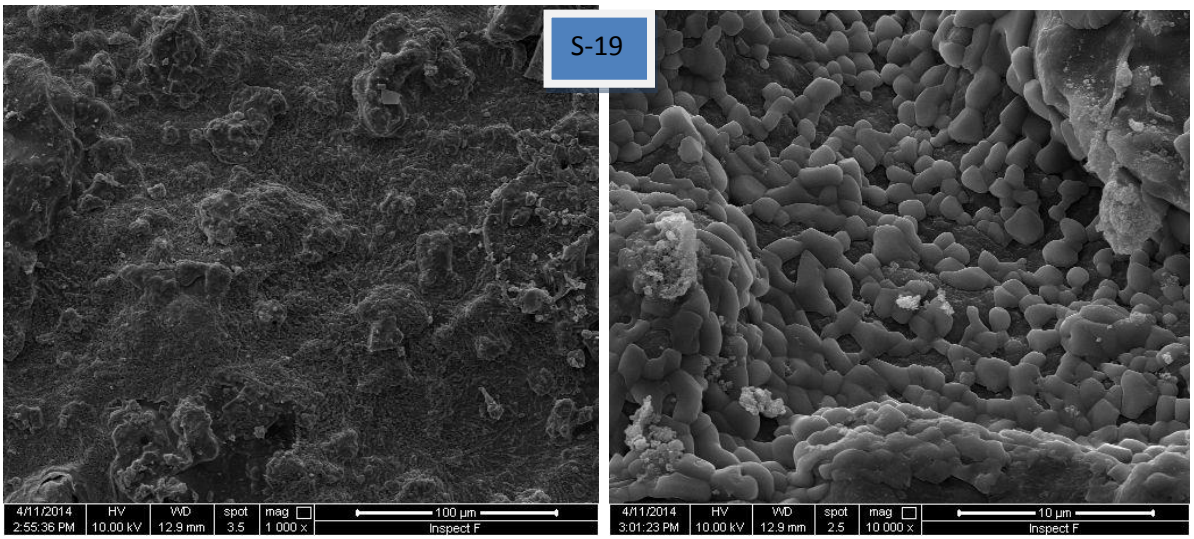
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100 µm
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WD 12.9 mm
spot 2.5
mag 10 000 x
10 µm
Inspect F

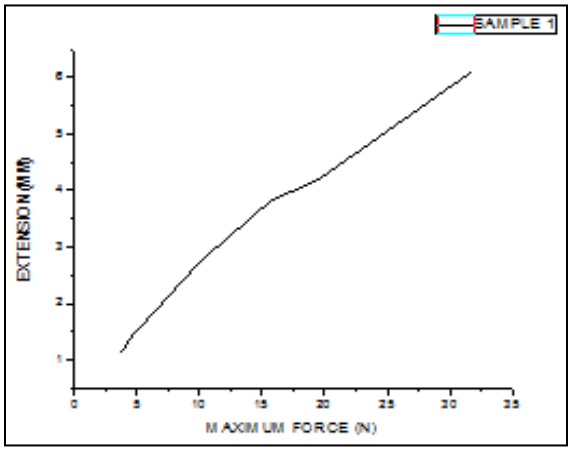
4.3. Tensile Strength

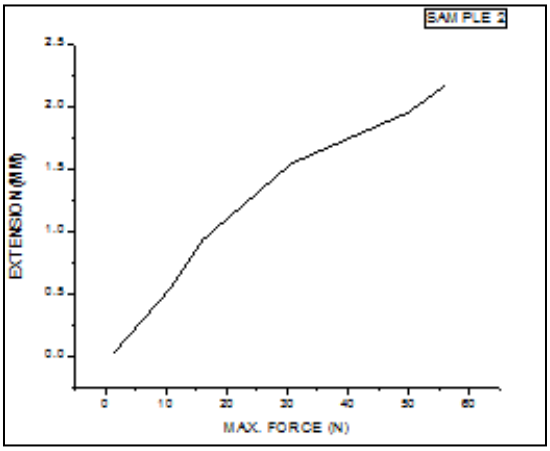
The maximum force of the samples was found out for sample 6 at 0%, 0.25%, 0.5% weight Hydroxyapatite as well as the maximum elongation. The table below represents the values of the tensile strengths obtained for Sample 6 with varying HAP content.

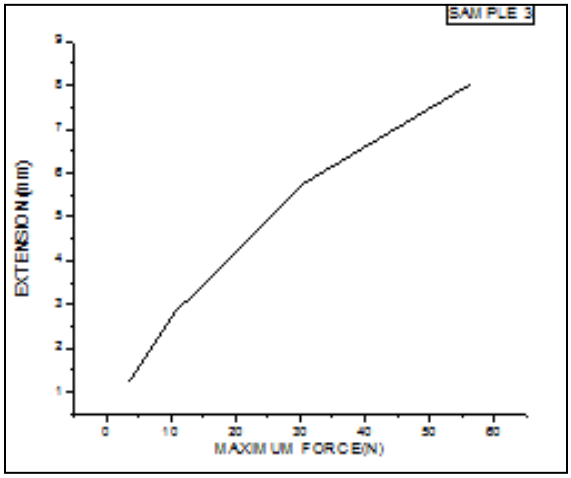
Sl. No	Composition	Extension (mm)	Tensile Strength (N/mm^2)
1	0% HAP	6.096	11.32
2	0% HAP	2.173	24.47
3	0.25% HAP	8.019	22.52
4	0.25% HAP	5.919	16.92
5	0.5% HAP	8.219	6.44
6	0.5% HAP	10.298	3.8

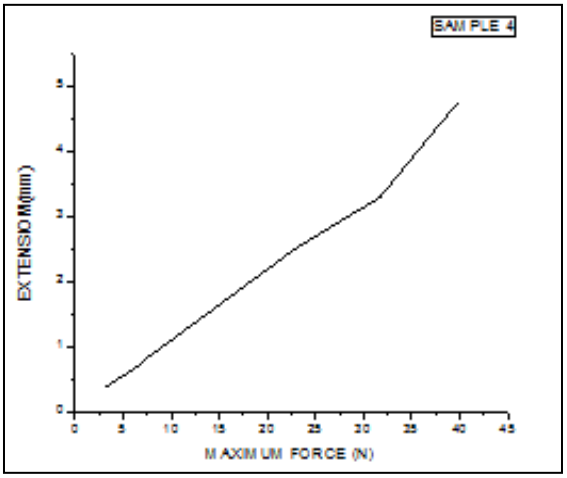
From this, we can infer that the tensile strengths of Samples 6 containing 0.5% HAP has reduced drastically. The samples containing 0% HAP has a variation which may be because of processing defects. Samples containing 0.25% HAP showed good tensile strength results that are near to the values of human skin strength. This indicates that it can be used for skin engineering

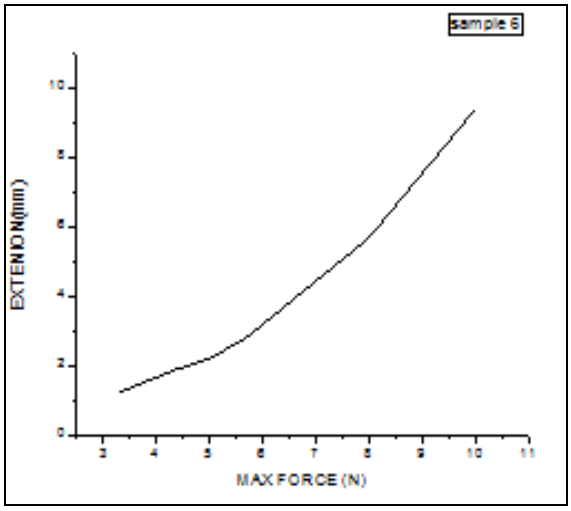
purposes. The graphs below showed the elongation versus the maximum force for the various samples.

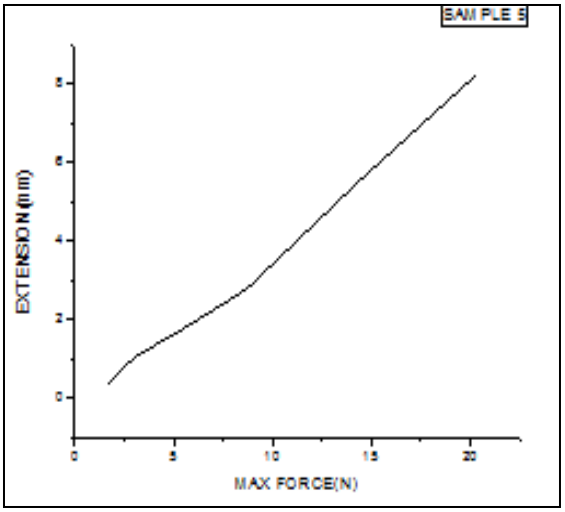








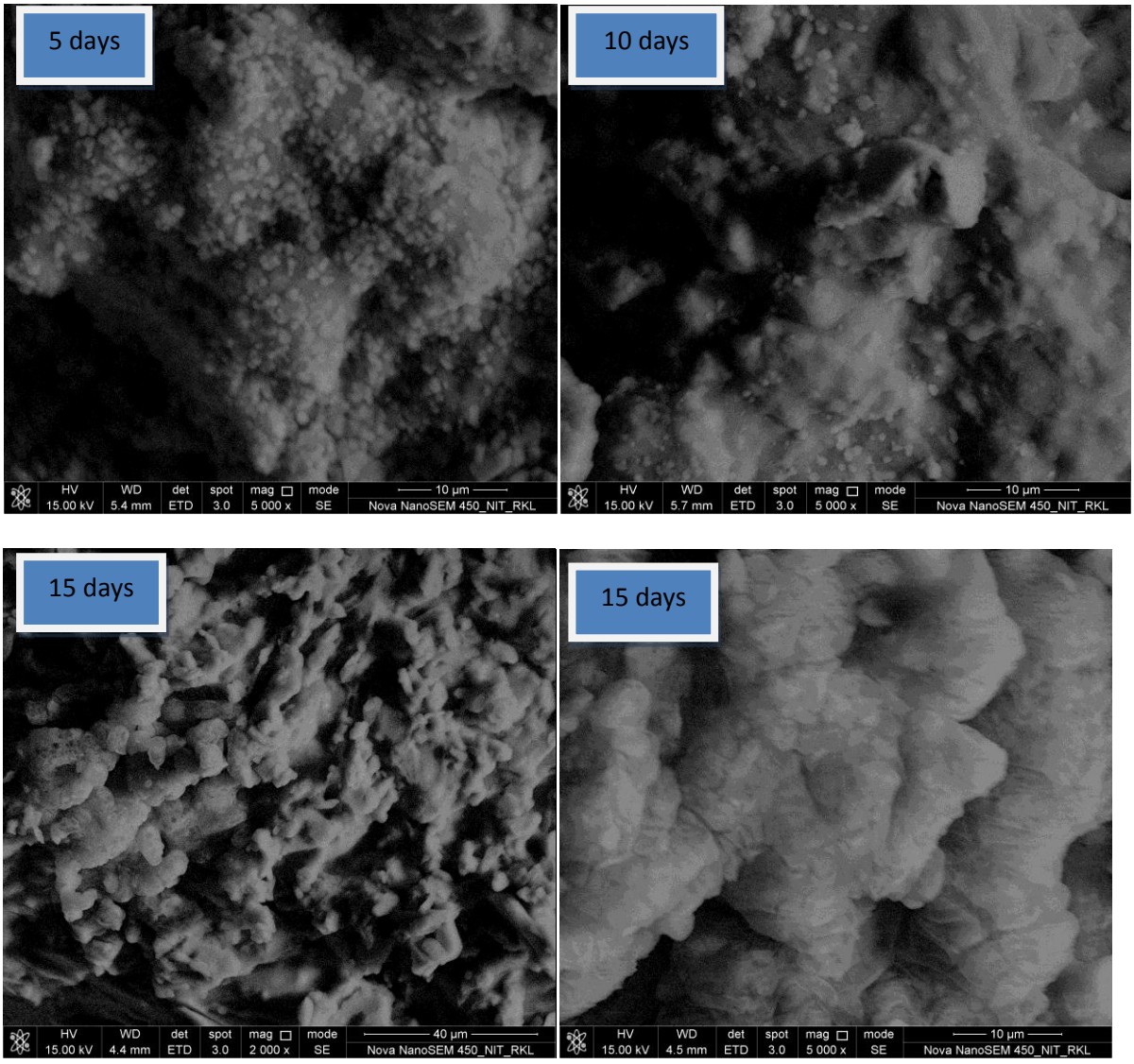




These graphs showed that the composite bear a good elastic property. Detailed process optimization can increase the tensile strength good enough to be used as human skin. Also Sample 3 shows the best results in term of tensile strength and intermediate extension showing that it can be used for biomedical applications.

4.4. Bioactivity

Bioactivity of the Sample 5 was done by the FESEM after 15 days. HAP formation was seen on the surface of the film showing that the film was bioactive. The images for the sample 5 showing the bioactivity of micro thin composites after 5 days, 10 days and 15 days are shown below. The figures below showed that there was a significant growth of HAP particles on the surface of the films at 10 µm magnification. Thus the films are bioactive and can be used for tissue engineering purposes and wound healing operations.

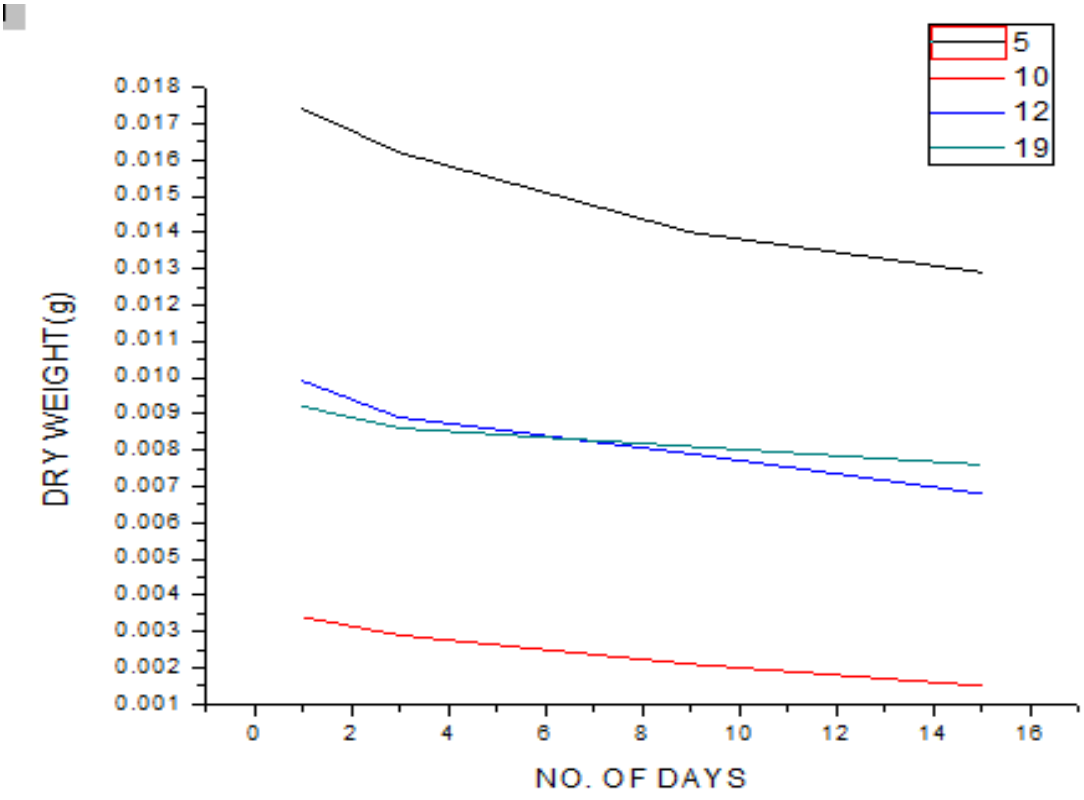


4.2.Bio-degradability

After dipping into the tris buffer solution for 15 days, the results came positive. There was a 25.86% decrease in dry weight in Sample 5, a huge 55.88% decrease in sample 10, a 31.31% decrease in sample 12 and a 17.39 % decrease in sample 19. The table of results for the samples having dry weight with respect to number of days is given below.

Sample No.	Dry weight(1 day)	Dry weight(3 days)	Dry weight(9 days)	Dry weight(15 days)
5	0.0174	0.0162	0.0140	0.0129
10	0.0034	0.0029	0.0021	0.0015
12	0.0099	0.0089	0.0079	0.0068
19	0.0092	0.0086	0.0081	0.0076

The graph of the different samples for dry weight versus number of days dipped into the tris buffer solution was plotted and hence given below:



Chapter-5

Conclusion

In the present study successful synthesis of microthin composites has been done. Physico-chemical and biochemical characterizations are carried for the micro-thin films showing positive results. XRD analysis shows that the crystallinity of the composite changed with respect to the HAP content. The pore size, particle distribution and homogeneity is also analysed by FESEM. Microstructural analysis depicts the distribution of HAp to be homogeneous and has high porosity for tissue ingrowth. The pore size can be further controlled using a pore-former. Although alginate has sufficed the role of a dispersant, further dispersant can be used to increase the homogeneity and improve the microstructure. The tensile strength is found to be 22.52N/mm^2. As the strength of the human skin is near to the value obtained for composite film, therefore, it can be inferred that these composites can be used for skin biomedical applications. The bioactivity and biodegradability shows that it can be used for tissue engineering applications. Further research on these bio-composites is an interesting field of work to understand the cell growth and cell adhesion to the tissue.

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